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ScienceDirect
Theriogenology 70 (2008) 1304–1309
Theriogenology
www.theriojournal.com

Swine gene banking: A quality control perspective on collection, and analysis of samples for a national repository ☆

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Abstract

The National Animal Germplasm Program (NAGP) is developing a national repository for germplasm (semen, oocytes, embryos, blood, DNA, tissue) for all agricultural species in the US. Currently, the swine collection consists of 127,479 samples from 886 boars representing 20 major, minor and composite populations. Cryopreservation per se is not an impediment to program success. Rather, the greatest difficulties encountered are in determining the quality of the samples pre- and post-thaw. Robust, broadly applicable, and cost effective quality control methodologies need to be developed and implemented. This overview of the NAGP will discuss the approaches used for cryopreserving boar semen samples, overcoming the challenges of assessing sample quality, and moving toward a quality control strategy.
Published by Elsevier Inc.

Keywords: Germplasm; Boar spermatozoa; Cryopreservation

1. Introduction

The USDA-ARS National Animal Germplasm Program (NAGP) was created to preserve agriculture and aquaculture species in the US due to the decrease in genetic diversity caused by economic pressures [1], and to protect this resource from potential disease outbreaks. Individuals are chosen, sampled (semen, eggs, embryos, DNA, blood, tissue) and cryopreserved for indefinite storage with the intent of creating a collection of material that can be used for repopulation, expanding the genetic base of a breed, as well as research. The germplasm collection is available to the public and private sectors. In developing the collection a significant task is maintaining and evaluating the quality of the boar semen samples (specifically for purposes of this manuscript) during collection, transportation, cryopreservation and post-thaw examination. Through NAGP and its associated species committees research on cryopreservation methodologies that will improve the post-thaw quality of cryopreserved material stored in the repository is conducted. This manuscript addresses the quality control aspects of cryopreserving boar semen, as it pertains to gene banking material for the NAGP, and present recent research directed at better evaluating boar sperm and improving its post-thaw quality.

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0093-691X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.theriogenology.2008.06.012

2. Program overview

2.1. Sample selection and acquisition

The NAGP is designed to collect samples from the major, minor and composite breeds of swine currently in the US [2]. Individual boars are chosen by cluster analysis which evaluates their pedigree data, provided from breed organizations, with the other males in the breed and identifies ''families'' of animals within a breed which should then be sampled [3]. This methodology enables the NAGP to capture the diversity of genetics across a breed and minimizes the repetition of males/genetics in the repository.

3. Semen processing

3.1. Collection, dilution, and shipping

Samples are sent to the NAGP in two forms; diluted semen or whole testes. The diluted semen samples are collected using the hand-glove technique and the sperm rich fraction is diluted (1–3; volume to volume) in Androhep Plus (Minitube of America, Verona, WI, USA) at 37 °C. The diluted sample is aliquoted into disposable centrifuge tubes, cooled to 23 °C and placed in a shipping container to cool and maintain the samples at 15 °C for overnight transport to the NAGP laboratory. Upon arrival the samples are centrifuged at 800 × *g* for 12 min at 15 °C and the supernatant is removed. The sperm concentration of the pellet is then determined using spectrophotometry and the samples are diluted to 300×10^6 sperm/mL in BF5 cooling extender [2]. The diluted samples are then placed in a beaker containing 15 °C water and cooled to 5 °C over 2–2.5 h. The samples are then diluted drop-wise to 200×10^6 sperm/mL using BF5 freezing extender [4], loaded into 0.5 mL CBS straws (IMV Corporation, Minneapolis, MN, USA), and cryopreserved in liquid nitrogen vapor using a Minidigitcool UJ400 programmable freezer (IMV Corporation) with the following freeze rates: 5 °C to −8 °C at −20 °C/min; −8 °C to −120 °C at −69 °C/min; −120 °C to −140 °C at −20 °C/min. The samples are then plunged into liquid nitrogen for storage. Samples are thawed for 20 s in a 50 °C water bath.

Under special circumstances, if a boar is not capable of being collected, or the boar is not trained for semen collection and is intended for slaughter, the whole testes are sent to the laboratory for sperm harvesting. After excision from the boar the testes are placed in plastic bags, sealed, and shipped in the same shipping container as previously mentioned. The epididymides are then excised and flushed into a 50-mL centrifuge tube using a 20-gauge needle, syringe and Beltsville Thawing Solution [4]. The sperm samples are centrifuged, processed and cryopreserved in the manner previously described.

3.2. Quality control–sample evaluation

To achieve the best post-thaw quality results, it is necessary to freeze boar sperm samples as soon as possible after collection and equilibration. Thus, the determination of quality control in a repository setting is difficult because little information exists that documents the effects of holding time prior to cryopreservation on the quality and fertilizing potential of boar sperm. In the cryopreservation protocol just described (Section 3.1), semen sample quality is continuously monitored. After initial collection the sample is microscopically evaluated for gross motility when possible (performed at commercial studs but not always possible when samples are collected by private producers). Upon arrival at the NAGP laboratory, semen pH and temperature are recorded. Our observations suggest that, as could be expected, when the temperature is too high (above 18 °C) or too low (below 12 °C) the pre-freeze and post-thaw motility/quality is deleteriously affected. Furthermore, the fluctuations in pH may also be detrimental to the post-thaw quality of boar sperm samples. The pH is normally maintained in the range of 6.6–7.2 in Androhep Plus when received at the NAGP. The incidence of samples arriving with a pH outside this range is rare (<1%). In the instance of the high pre-freeze pH samples (pH > 7.8) the samples often have very low post-thaw motility (less than 10% total motility) even when the initial motility may have been in an acceptable range (e.g. >70% via technician observations). The elevated pH may be due to a number of factors such as incorrect media preparation or generally poor sample quality, but regardless, the increase in pH may be inducing capacitation and hyperactivation [5] and consequently decreasing the post-thaw quality.

The next quality control aspect evaluated is the motility of the sperm sample. The NAGP performs pre-freeze and post-thaw motility analysis using computer-assisted sperm analysis (CASA; Hamilton Thorne Research, Beverly, MA, USA). For each sample, a minimum of seven fields and 1000 sperm are evaluated using a Leja Standard Count 2 Chamber Slide with a 20-μm depth (Spectrum Technologies, Healdsburg, CA). The CASA is set up with the following parameters; 30 frames acquired, frame rate of 60 Hz, minimum contrast

Table 1
Post-thaw motility characteristics of the 886 boar semen samples cryopreserved and stored at the National Animal Germplasm Program repository

Parameter	Mean	S.D.
Motility (%)	29.4	15.7
Progressive motility (%)	13.1	10.0
Cell area (μm^2)	8.9	0.93
Path velocity, VAP (μm/s)	69.1	12.6
Linearity (%)	38.5	5.7
Straightness (%)	71.2	7.0
Lateral amplitude, ALH (μm)	6.9	1.1
Elongation (%)	48.5	3.6
Track speed, VCL (μm/s)	136.4	24.8
Progressive velocity (μm/s)	50.7	10.1
Beat frequency, BCF (Hz)	34.4	4.1

of 55, minimum cell size of 5 pixels, VAP cutoff of 20 μm, progressive minimum VAP cutoff of 45 μm/s, VSL cutoff of 5 μm/s, static head size of 0.53–4.45, and magnification of 1.89, 37 °C stage. Post-thaw motility statistics for the boar semen samples in the repository are presented in Table 1.

3.3. Quality control–sample physical safety

To facilitate the collection, processing, storage and the physical security of the samples the CBS 0.5 mL straw, MAPI system (straw loader/sealer/labeler), and associated programmable freezer (IMV Corporation, Minneapolis, MN) were selected to be used by the NAGP for freezing all samples. This system is advantageous because it enables a single person to load, seal, and custom label 600 straws per hour while performing other tasks such as diluting an additional sample of boar semen with freezing extender. Additionally, the straws, when heat-sealed in the MAPI, are not permeable to external contamination from impurities in the liquid nitrogen or thawing water bath and the straws remain flexible in liquid nitrogen thus eliminating breakage and loss of valuable samples. Furthermore, the automated freezer ensures repeated uniform freezing of samples (1400 per freeze) while enabling the user to alternate between different customizable freezing protocols. Following cryopreservation the samples are stored in liquid nitrogen tanks in CBS goblets and Visotubes (IMV Corporation) for easy identification, inventory management and retrieval.

3.4. Quality control–cryopreservation decisions

The previous sections discussed the quality control aspects (pH, temperature and motility) that are presently used for evaluation. When a sample arrives at the NAGP the described measurements are performed and a decision is made whether the sample qualifies for cryopreservation. Preferably all boars are collected and frozen twice with at least a 2-week interval between collections. The number of boars and straws per boar is modified based on whether a boar belongs to a major or minor breed, or a commercial composite population. For major breeds then 155 straws per boar of interest are cryopreserved if the pre-freeze total motility is greater than 30%; otherwise the sample is discarded and an additional sample is acquired from the producer/stud. In the instance of minor breeds, no more than 500 straws per ejaculate are cryopreserved, provided there is some motility in the sample. If a complete lack of motility is present in a minor breed sample only 25 straws per ejaculate are frozen for DNA extraction purposes and an additional ejaculate will be requested. Minor breeds are generally not in commercial studs or within reasonable distance of someone who knows how to train and collect boars. Therefore, it can be difficult to get additional samples so the quantity of straws obtained from each ejaculate is maximized.

4. Research

Research activities are focused upon the improvement and/or prediction of the quality of frozen–thawed samples, and understanding the physiology of cells and tissues undergo cryopreservation. This involves applied and basic science where new cryopreservation protocols or methods of quality evaluation are examined to determine if they should be implemented into the NAGP protocols and exploration of the physiology and cryobiology associated with the cells and tissues preserved in the repository. To this end, the following are examples of research being performed at the NAGP to improve our understanding of the quality/fertility of samples following cryopreservation and to increase our knowledge of the cryopreservation process.

4.1. Ubiquitination of boar sperm

4.1.1. Ubiquitin overview

Ubiquitination is an apoptotic mechanism used for labeling and destroying defective cells by attaching a small protein (ubiquitin) to the plasma membrane. The theory behind ubiquitin's role in spermatogenesis is that spermatozoa are ubiquitinated to eliminate imperfect cells by the proteasome in the testis [6] and by phagocytosis in the epididymis [7], and as a result, higher quality sperm will be present in the ejaculate

than if this mechanism was not in place. Ubiquitinated sperm have been detected in boars [8,9], humans [10], stallions [6], and bulls [7] and correlated with infertility in each species. Contrary to findings which associated ubiquitination with poor sperm quality, Muratori et al. [11] reported that in human sperm, ubiquitination is indicative of higher quality as determined from motility and morphology analyses. Despite the work of these investigators, there is a significant gap in our knowledge concerning the temporal effects of ubiquitination therefore; we lack a complete evaluation of the capacitation and acrosome reaction processes.

Cryopreservation success is largely affected by the plasma membranes' ability to respond to fluctuating environmental conditions associated with cryopreservation media, dilution as well as decreasing and increasing temperatures. As ubiquitin is a protein bound to the plasma membrane of the sperm, it could potentially influence the cryopreservation process by altering the membrane stability/fluidity of a spermatozoon. Sutovsky et al. [7] reported that the dilution of sperm in cryopreservation media and the cryopreservation process do not alter ubiquitin labeling but this leads one to wonder if cryopreservation success can be influenced by ubiquitination, as only post-thaw results from many of the previously mentioned experiments were reported.

Based on this information the goal was to determine: first, if ubiquitination influences sperm function (motility, mitochondrial function) and structure (membrane integrities, membrane fluidity) prior to and following cryopreservation; and second, if ubiquitination affects frozen–thawed sperm physiology (capacitation, acrosome reaction, plasma membrane integrity). If ubiquitination impacts sperm physiology then ultimately these analyses could be used as a quality/fertility predictor.

4.1.2. Materials and methods

4.1.2.1. Experimental design. Semen was collected from 12 boars (one sample per boar) and cryopreserved as described previously. Aliquots of the sperm samples (fresh, prior to cryopreservation, and post-thaw) were evaluated for motility, as described earlier, and flow cytometrically (CyAn-ADP flow cytometer, Dako-Cytomation Inc., Fort Collins, CO, USA) for plasma membrane and acrosomal membrane integrity [12], mitochondrial function [13], intracellular calcium [14], membrane fluidity/capacitation [15], and ubiquitin [7,10]. In addition, plasma membrane/acrosomal membrane integrity and intracellular calcium were determined at 15 min intervals up to 120 min while being incubated in 10 mM calcium TALP [16] to evaluate the ability of frozen–thawed boar sperm to capacitate and acrosome react.

4.1.2.2. Statistics. The ANOVA procedure of SAS [17] was used to identify differences in the percentage of ubiquitinated sperm for the treatment effect of cryopreservation status (fresh or frozen–thawed) of boar sperm. Mean differences were separated using the Student–Newman–Keuls test. Percentage data were arcsine transformed.

Fresh or frozen–thawed percentages of sperm from the motility, plasma membrane integrity, live acrosomal membrane integrity, and membrane fluidity analyzed for correlations (PROC CORR) with the percentage of fresh or frozen–thawed ubiquitin positive sperm (independent variables) [17].

Because of the repeated measures aspect of the capacitation analyses, the mixed procedure [17], containing the fixed effects of frozen–thawed ubiquitination and time and their interactions was used. Boars were incorporated into the model as random effects. The dependent variables included plasma membrane integrity, acrosomal integrity, or intracellular calcium when sperm samples from 12 boars (random effects) were induced to capacitate over 120 min. Multiple regression analysis was used to regress the percentage of plasma membrane intact, high intracellular calcium, or live acrosome reacted sperm on time and the percentage of ubiquitinated sperm [17].

4.1.2.3. Results and discussion. Differences in pre-freeze and post-thaw levels of boar sperm ubiquitination were observed using flow cytometric analysis which demonstrates that boar sperm have significantly less ubiquitin bound to the membranes following cryopreservation (Table 2), most likely due to the physical stress of cryopreservation which has been documented as being capable of decreasing sperm plasma membrane

Table 2
Characteristics of fresh and frozen–thawed boar sperm that were analyzed for ubiquitination using flow cytometry and for motility characteristics using CASA

Sperm characteristic	Fresh	Frozen–thawed	S.E.M.
% Ubiquitinated	29.4[a]	20.0[b]	2.7
Minimum ubiquitination (%)	11	5	n/a
Maximum ubiquitination (%)	49	46	n/a
Total motility (%)	75[a]	13[b]	4.6
Progressive motility (%)	40[a]	5[b]	3.7
VSL (μm/s)	61.2[a]	57.3[b]	1.1
VAP (μm/s)	100.6[a]	77.4[b]	3.0

[ab]Different superscripts indicate row differences ($P < 0.0001$).

protein content. The percentage of frozen–thawed ubiquitinated sperm was significantly correlated ($P < 0.05$) with frozen–thawed progressive velocity (VSL; $r = 0.59$) and path velocity (VAP; $r = 0.58$). Differences between fresh and frozen–thawed VAP were observed but not for VSL (Table 2). All other sperm characteristics analyzed were non-significant.

Analysis of the percentage of boar sperm demonstrating high intracellular calcium during the capacitation analysis did not result in identification of a significant model. However, evaluation of proportions of live acrosome reacted sperm (FITC positive; ($y = 29.1 + (0.1 \times \text{time}) + (-0.25 \times \%$ of ubiquitin positive sperm))) or proportions of boar sperm maintaining plasma membrane integrity ($y = 36.2 + (0.034 \times \text{time}) + (0.237 \times \%$ of ubiquitin positive sperm)) during the 120-min of capacitation incubation resulted in identification of significant models ($P < 0.05$).

In these analyses, ubiquitination of sperm is related to higher motility, velocities (although not at hyperactivation levels [5]), and plasma membrane integrity after thawing, consequently greater quality (overall). Furthermore, this research also demonstrates that when induced to capacitate, ubiquitinated sperm undergo capacitation at a slower rate and simultaneously maintaining their plasma membrane integrity. Recently though, results of a fertility trial encompassing 1754 sow AIs from 13 boars demonstrated that there is a correlation ($r = -0.31$; $P < 0.05$) between sperm ubiquitination and farrowing rates [9]. If the findings of Lovercamp et al. [9] and the present study are valid, then the traditional sperm quality assessments (motility, plasma membrane integrity, etc.) need to be reevaluated as to their value.

4.2. Plasma membrane protein analysis

The success of cryopreservation is attributed to plasma membrane lipid content. However, membrane protein content, the other major membrane component, has been ignored for the purpose of determining the permeability of a membrane and cryopreservation success possibly because of a lack of available tools and methodologies to do so. Thus, a starting point for research, in which the protein content of the entire sperm plasma membrane can be identified and compared, would offer insight into the influence that total plasma membrane protein may have on boar sperm physiology and cryobiology as a whole. Therefore, as exploratory research, the following experiment was performed.

4.2.1. Materials and methods

Semen samples from composite population boars ($n = 17$) were collected and cryopreserved as described previously. Fresh (pre-freeze) and frozen–thawed aliquots were analyzed for motility using CASA. Boar sperm samples were also analyzed flow cytometrically for protein content using Rhodamine 640 (Exciton, Dayton, OH) [18] at room temperature (23 °C), after dilution with BF5 cooling extender and cooling to 5 °C over 2 h, then after addition of the BF5 freezing extender, and after cryopreservation and thawing.

4.2.2. Statistics

The boars were placed into two groups based on whether their sperm sample had an increase or decrease in relative mean protein fluorescence of the sperm population compared to their analysis at 5 °C after dilution with the BF5 freezing diluent. The PROC GLM [17] was used to test for differences in boar sperm protein fluorescence of the two groups (independent variable) prior to and following freezing, as well as for motility characteristics. Mean differences were separated using Student–Newman–Keuls mean separation test [17].

4.2.3. Results and discussion

Differences in relative mean fluorescence by group were observed over the cooling and cryopreservation treatments (Fig. 1). Significant differences in post-thaw progressive motility (36.0 and 21.3%), beat cross-frequency (33.9 and 36.6 Hz), and lateral head amplitude (6.6 and 5.8 μm) evaluated immediately after thawing were detected as well for protein groups one and two, respectively.

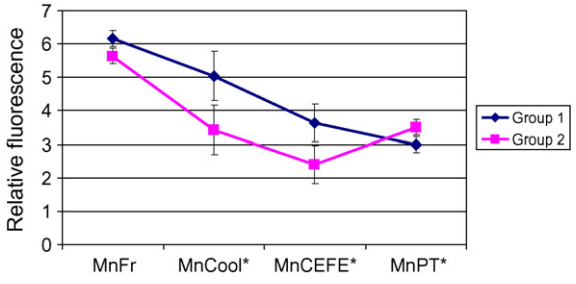

Fig. 1. The relative fluorescence of boar sperm analyzed to determine the plasma membrane protein content using Rhodamine 640 (protein) and Yo-Pro 1 (plasma membrane integrity). Differences in mean fluorescence between groups and by evaluation environment are indicated by an asterisk (*). Mean fluorescence was evaluated at: MnFr = 23 °C; MnCool = after dilution in BF5 cooling diluent and cooling to 5 °C; MnCEFE = the MnCool sample after dilution with the BF5 freezing diluent fraction at 5 °C; MnPT = at 23 °C following cryopreservation and thawing.

Potentially, these findings could have relevance to a number of areas of boar sperm physiology and cryobiology as a whole. First, the changes in the exposed protein (either native plasma membrane protein or added extracellularly from the cryopreservation diluent) levels may illustrate a plasma membrane protein rearrangement associated with the diluent and, or in combination with the BF5 diluent (egg yolk, detergent, etc.) during cooling and cryopreservation. This ability to rearrange during cooling and cryopreservation may result in a sperm's ability to respond to and survive, as illustrated by the difference in post-thaw progressive motility, the physical insults associated with the processes. In addition, because of the differences in types of post-thaw motility, the identification of exposed protein may also serve as a potential indicator of post-thaw quality as Group 1 appears to be in a hyperactivation state [5]. More analyses are needed, and are ongoing, to understand the actions of and identification of sperm plasma membrane protein during the cryopreservation processes and to fully comprehend its role in sperm physiology.

5. Future directions for quality control and conclusions

Quality control and determining the quality of cryopreserved samples is essential for developing a successful national repository. While quality control measures are currently in place at the NAGP we are striving to expand their scope and to develop additional measures using available technology to assess sample quality thoroughly. In the near future we will compare and implement a more thorough usage of the CASA with the goal of developing a model to value the sperm samples beyond the motility descriptors currently reported. This modeling will also incorporate flow cytometry, utilizing assays like the ubiquitin and protein analyses, to expand the scope of information evaluated. The flow cytometry assays incorporated will need to be determined based on which assay can provide the greatest amount of information about a sample while minimizing costs, preparation time for analysis, and post-analysis interpretation.

Acknowledgements

The author gratefully acknowledges the assistance of Dr. Harvey Blackburn, Ms. Carrie Welsh, Ms. Andra Cox, and Mr. Scott Spiller for their assistance with this manuscript and all the activities with which we are involved at the NAGP.

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